Mitochondrial DNA polymerase $\gamma$ is expressed and translated in the absence of mitochondrial DNA maintenance and replication

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ABSTRACT

Mitochondria are essential organelles in all eukaryotic cells where cellular ATP is generated through the process of oxidative phosphorylation. Protein components of the respiratory assembly are gene products of both mitochondrial and nuclear genes. The mitochondrial genome itself encodes several protein and nucleic acid components required for such oxidative phosphorylative processes, but the vast majority of genes encoding respiratory chain components are nuclear. Similarly, the processes of replication and transcription of mitochondrial DNA rely exclusively upon RNA and protein species encoded by nuclear genes. We have analyzed two key nuclear-encoded proteins involved in mitochondrial DNA replication and transcription as a function of the presence or absence of mitochondrial DNA. Mitochondrial DNA polymerase (DNA polymerase $\gamma$), the nuclear-encoded enzyme which synthesizes mtDNA, is expressed and translated in cells devoid of mitochondrial DNA itself. In contrast, mitochondrial transcription factor A protein levels are tightly linked to the mtDNA status of the cell. These results demonstrate that the DNA polymerase $\gamma$ protein is stable in the absence of mitochondrial DNA, and that there appears to be no regulatory mechanism present in these cells to alter levels of this protein in the complete absence of mitochondrial DNA. Alternatively, it is possible that this enzyme plays an additional, as yet undefined, role in the cell, thereby mandating its continued production.

INTRODUCTION

Energy-generating mitochondria contain their own double-stranded circular genome, mitochondrial DNA (mtDNA). In mammals, mtDNA constitutes ~1% of total cellular DNA. MtDNA copy number is maintained remarkably constant, ranging from 1 to 10 000 copies per cell, a signature of the particular cell or tissue type (1). Replication and transcription of mtDNA requires the participation of several nuclear-encoded enzymes, some of which consist of both protein and nucleic acid components (2). Copy number control then must depend upon careful orchestration of processes which regulate mitochondrial nucleic acid metabolism, soliciting the cooperation of both nuclei and mitochondria.

Several notable features distinguish the replication of mtDNA and chromosomal DNA. In contrast with chromosomal (nuclear) DNA, mtDNA synthesis does not appear to be restricted to any particular phase of the cell cycle (3). Moreover, mtDNA may be replicated more than once or not at all within a given cell generation time (4). Replication of chromosomal DNA is heavily monitored and corrected through elaborate DNA repair mechanisms, whereas limited DNA repair occurs within the mitochondrion (2). In keeping with the different requirements for synthesis of mtDNA, replication and transcription of the organellar genome is thought to occur within the mitochondrial matrix through the activity of the mitochondrial-specific replication and transcription machineries. Both mtDNA polymerase (mtDNA polymerase $\gamma$) and mitochondrial RNA (mtRNA) polymerase are encoded by nuclear genes in vertebrates (Ropp and Copeland, submitted; Garman and Clayton, unpublished observations), as are all known transcription factors which interact with DNA sequences present in nuclear or mitochondrial genes involved in either oxidative phosphorylation or mtDNA replication and transcription processes (5–7).

Mammalian cells in culture can be completely and irreversibly depleted of mtDNA by long-term exposure to the intercalating agent ethidium bromide (EB) (8). Alternatively, short-term exposure to very low concentrations of EB leads to reversible inhibition of mtDNA synthesis, and consequent depletion of mtDNA (9). Cells devoid of mtDNA ($\rho^0$ cells) can be readily maintained in culture, given the presence of exogenous pyruvate and uridine (8). $\rho^0$ cells exhibit uridine auxotrophy due to a deficiency in respiratory chain-dependent dihydroorotate dehydrogenase (10) and rely exclusively on glycolysis for energy. In the presence of pyruvate and uridine, however, the growth rate of $\rho^0$ cells approaches that of the parental cell line from which they were derived (11). $\rho^0$ cells are incapable of producing energy through oxidative phosphorylation due to the absence of all mitochondrially-encoded proteins, which together represent a

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relative small percentage of all proteins present in the inner membrane enzyme complexes (12). Apparently the remaining contingent of nuclear-encoded respiratory subunits is sufficient to maintain a nearly normal membrane potential (13).

Previous studies in the yeast *Saccharomyces cerevisiae* have employed *p*^0^ cells as a model system to investigate potential compensatory responses elicited by nuclear genes in the absence of mtDNA (14–17). Results from such studies suggest that, at least in single-cell eukaryotes, there may exist some forms of genetic communication between the mitochondrial and nuclear organelles. While *p*^0^ strains of *S. cerevisiae* have been used extensively over the years to study factors affecting mtDNA metabolic processes, significantly less is known concerning mammalian *p*^0^ cells. A few recent studies, however, have employed subtractive hybridization strategies to investigate molecular differences between *p*^0^ and *p*^+^ cells (13,18).

In an effort to understand further interorganellar communication between nuclei and mitochondria, we sought to analyze certain nuclear-encoded proteins involved in maintenance of mtDNA copy number in either the presence or absence of mtDNA. Our results suggest that the presence, and presumed activity, of proteins which play a role in the replication and transcription of mtDNA appear not to be universally dependent on the mtDNA status of the cell.

**MATERIALS AND METHODS**

**Cell culture maintenance**

143B (TK−) human osteosarcoma cells (ATCC CRL 8303, kindly provided by Dr Eric A. Shoubridge) were maintained in a humidified 5% CO₂ atmosphere in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (Gibco-BRL, Bethesda, MD), 1.25 µg/ml gentamycin (Sigma, St Louis, MO), 50 µg/ml uridine and 100 µg/ml (333 µM) BrdU (both from US Biochemicals, Cleveland, OH). 143B (TK−) cells containing (p^+^) or lacking mtDNA (p^0^) were cultured under identical conditions. Two independent *p*^0^ clones (#4 and #14) were used and gave equivalent results in all experiments. HeLa human cervical carcinoma cells (ATCC CCL 2) were grown as monolayer cultures in DMEM (Gibco-BRL, Bethesda, MD) containing high glucose, L-glutamine and 110 mg/l sodium pyruvate, and supplemented with 5% FBS and 1.25 µg/ml gentamycin. For ethidium bromide (EB) treatments, HeLa cells were first cultured onto glass coverslips and then treated for 3 days with 20 or 100 ng/ml EB (Sigma), diluted into DMEM from a 1 µg/ml stock solution.

**Western blot analysis**

Whole-cell extracts from 143B (TK−) cells were prepared as previously described (19). Briefly, ~10^7^ cells were centrifuged and washed with phosphate buffered saline (PBS). Cells were then washed twice in 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl and 0.5 mM DTT, pelleted, and resuspended in 20 µl of 20 mM HEPES, pH 7.9, 25% glycerol, 0.42 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM FMSF, 1 µg/ml pepstatin A, 1 µg/ml leupeptin, 1 µg/ml aprotinin and 0.1% Nonidet P-40. The suspension was incubated on ice for 10 min, mixed briefly, and pelleted at 14,000 g. The lysed cell supernatant was diluted with 20–50 µl buffer D, containing 20 mM HEPES, pH 7.9, 20% glycerol, 50 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1 µg/ml pepstatin A, 1 µg/ml leupeptin and 1 µg/ml aprotinin. Protein concentrations were determined by the method of Bradford (20), and extracts were stored at −80°C. Extracts (10–20 µg) were resolved by SDS–PAGE, using 10% linear polyacrylamide gels. Prior to electrophoresis, proteins were denatured by boiling in SDS (0.5%) at 100°C for 5 min, and disulfide bonds in proteins were reduced by treatment with β-mercaptoethanol. Following electrophoresis, proteins were transferred to a nitrocellulose membrane by electroblotting for 1 h at 100 V in 6.25 mM Tris–HCl, pH 7.5, 1.3% glycine and 20% methanol. The membrane was blocked at 4°C for 15 h in TBS-T (20 mM Tris–HCl, pH 7.5, 136 mM NaCl, 0.1% Tween-20), supplemented with 5% non-fat dried milk. After washing briefly with TBS-T, the membrane was incubated at 25°C for 60 min with primary antibody [polyclonal anti-DNA polymerase γ pre-immune serum (both diluted 1:500 in TBS), or monoclonal anti-actin (1:2000 in TBS) (Amersham, Buckinghamshire, UK)]. After repeated washes with TBS-T, the membrane was incubated at 25°C for 60 min with secondary antibody [<HRP> swine anti-rabbit IgG (DAKO, Carpinteria, CA) or <HRP> sheep anti-mouse IgG (Amersham)]. The membrane was then washed repeatedly with TBS-T, and immunoreactive species were detected by autoradiography with chemiluminescence, according to the manufacturer’s instructions (Amersham UK, Buckinghamshire, UK).

**Immunocytochemistry**

Monolayers of 143B (TK−) and HeLa cells were seeded onto autoclaved glass coverslips and cultured in 60 mM plastic dishes (Becton Dickinson, Lincoln Park, NJ) in supplemented DMEM. Prior to fixation, cells were labeled *in vivo* with the mitochondrial membrane potential-sensitive dye Mitotracker™ (Molecular Probes, Eugene, OR). Briefly, cells were incubated in the presence of the dye (100 nM, diluted in DMEM) for 15 min at 37°C, followed by an identical incubation in DMEM, except in the absence of dye. Labeling and all subsequent steps were performed in the absence of light. Cells were then washed twice with PBS, pH 7.4. Cells were first fixed with 4% paraformaldehyde [diluted from freshly prepared 20% paraformaldehyde (Sigma)] for 10 min at 25°C, followed by fixation/dehydration with –20°C acetone. Cells were rehydrated for 3 min (25°C) to 20 h (4°C) in PBS. Both primary and secondary antibodies were diluted in PBS/0.1% BSA at the following concentrations: anti-DNA polymerase γ (1:250), pre-immune serum (DNA polymerase γ (1:250), anti-mtTFA (1:1000), <Cy5>anti-rabbit IgG (1:500). Primary antibody incubations were performed for 60 min at 25°C, and secondary antibody incubations were performed for 30 min at 25°C. Cells were washed repeatedly between primary and secondary antibody incubations as well as after the secondary antibody incubation period. Finally, cells on coverslips were mounted onto glass slides with Cytoseal 60 mounting medium (Stephens Scientific, Riverdale, NJ). Microscopy was performed using a BioRad MRC 1000 laser-scanning confocal microscope, equipped with CoMOS imaging software. Digital images were merged using Adobe Photoshop software (Adobe Systems Inc., Mountain View, CA).

**Ribonuclease protection assay**

Total RNA was isolated from 143B (TK−) cells (*p*^0^ and *p*^+^) with TRIzol reagent (Life Technologies) as previously described (21). Non-isotopic ribonuclease protection assays were performed with the BrightStar BIOTINscript, RPA II and BrightStar.
BioDetect kits (Ambion, Austin, TX). The ribonuclease protection assay biotin-labeled probe was prepared from the human DNA pol γ plasmid cDNA clone K12 (Ropp and Copeland, submitted) as follows. The K12 clone was digested with KpnI, and the 3.1 kb fragment was isolated. This fragment contains the pBluescript vector and a 184 bp portion of the DNA pol γ cDNA. The fragment was religated to generate DK12184BS. This was submitted as follows. The K12 clone was digested with pBluescript vector and a 184 bp portion of the DNA pol γ cDNA was used to amplify the polymerase domain of the human gene (Ropp and Copeland, submitted). The amplified products were subcloned into the E.coli expression vector pGEX2T (Pharmacia, Uppsala, Sweden) to express the DNA pol γ peptide, GT14 to L1061, as a glutathione-S-transferase fusion protein (CD-7). The CD-7 antigen was expressed and purified from E.coli DH5α as described (23). Anti-CD7 antibodies were purified over protein-A agarose as described (24).

RESULTS
Steady state DNA polymerase γ message levels
In order to investigate potential intracellular communication links between mitochondria and nuclei, we employed a previously characterized human cell line [143B(TK−)] with (p+) or without (p−) mtDNA (11). 143B(TK−) p− cells were isonuclear with the parental 143B (TK−) cell line since they were generated from the parental cells through long-term exposure to EB as previously described (8), and exhibited characteristic uridine- and pyruvate-dependence for growth in culture (see Materials and Methods). To confirm that long-term exposure of the 143B (TK−) cells to EB had completely depleted the cells of mtDNA, total cellular DNA was extracted from the cells and probed simultaneously for both mtDNA (mtLeu) and genomic DNA (DNA pol γ) by PCR (Fig. 1). The mtLeu gene was chosen as a probe because it lies in an area of frequent deletions and/or duplications (25), however repeat experiments with additional mtDNA probes produced identical results (data not shown). MtDNA was only detectable in the parent 143B (TK−) cell line as well as in a control cell line (LS180), both of which are p+ (Fig. 1, lanes 1 and 4). The two p− isolates did not contain any detectable mtDNA even though approximately two to three times more of these samples were loaded than the p+ sample, based on the intensity of the genomic signal (Fig. 1, lanes 2 and 3). In addition, these cells exhibited a phenotype (pyruvate- and uridine-dependency) expected of p− cells.

Steady state message levels of mRNA transcribed from the nuclear-encoded mtDNA polymerase gene (DNA pol γ) were determined for p+ and p− cell lines to determine whether or not the expression of this gene might be affected by the presence or absence of mtDNA (Fig. 2). Initial attempts by Northern blotting procedures were unsuccessful at detecting DNA pol γ message even in the parent p+ cells, due to the low level of DNA pol γ mRNA. Therefore, we used ribonuclease protection assays to measure expression of DNA pol γ. As shown in Figure 2, message levels transcribed from DNA pol γ from both p+ and p− 143B (TK−) cell lines were approximately the same (Fig. 2, lanes 1 and 2 versus lanes 3–6). Integration of the band intensities indicated that there was no significant difference between DNA pol γ expression in p+ and p− cells. In addition, because the probe was used at saturating concentrations, we expected to find no appreciable difference upon addition of increasing amounts of probe (Fig. 2, lane 1 versus 2, 3 versus 4 and 5 versus 6). These results demonstrate that the absence of mtDNA does not influence the steady state level of the DNA pol γ message.
DNA polymerase ρ+ or γ protein appeared equivalent in whole-cell extracts from both blotting analysis revealed that overall levels of DNA polymerase and γ protein in 143B (TK−) cells. Western blot analysis with fractionated present at equal levels in ρ+ and ρo cells (arrowhead, Fig. 3, lanes 3 and 4). Previous studies have shown that the levels of certain proteins are elevated in ρo cells as compared with their parental counterparts (13). Equivalent amounts of protein were present in ρ+ and ρo cell extracts as revealed by nearly identical levels of actin protein (Fig. 3, lower panel).

We employed immunocytochemical analysis with laser-scanning confocal microscopy to confirm more definitively the subcellular localization of the DNA polymerase γ protein. Double labeling of 143B (TK−) ρ+ (Fig. 4a–f) and ρo (Fig. 4g–l) cells with a mitochondrial membrane potential-sensitive dye (Mito-tracker³, appearing red, Fig. 4b) and anti-DNA polymerase γ (see Materials and Methods, appearing green, Fig. 4a) revealed that the mtDNA polymerase γ is indeed present in mitochondria of these cells regardless of the mtDNA status of the cell. Merging of the two digital images allowed assessment of co-localization (appearing yellow, Fig. 4c). Approximately equivalent levels of DNA polymerase γ protein were detectable in ρ+ and ρo (Fig. 4c) 143B (TK−) cells. Control experiments with pre-immune serum revealed neither staining (Fig. 4d and j) nor co-localization (Fig. 4f and l) in either ρo or ρ+ cells. Note that as previously reported (13), mitochondrial membrane potential is relatively unperturbed despite the complete absence of mtDNA and, consequently, all mitochondrially-encoded proteins of the organellar membrane (Fig. 4k). These results indicate that the presence of mtDNA polymerase γ is not dependent on the concomitant presence of mtDNA.

The absence of mtDNA in ρo cells demands the absence of mtDNA replication. This has been confirmed by the complete lack of incorporation of the thymidine analog BrdU into 143B (TK−) ρo cell mitochondria (Davis and Clayton, submitted). Since ρo cells represent a long-term depletion of mtDNA, we were curious whether or not short-term depletion of mtDNA would exhibit any noticeable effect on levels of nuclear-encoded proteins involved in mtDNA copy number control, such as mtDNA polymerase γ and mitochondrial transcription factor A (mtTFA). Although EB is toxic to cells after approximately three cell generations (26), exposure to relatively low doses of EB for

**Figure 1.** Quantitation of mtDNA in ρ+ and ρo 143B (TK−) cell lines by PCR. PCR was performed as indicated in Materials and Methods. PCR products were resolved on an 8% sequencing gel containing 40% formamide and subjected to autoradiography. Lane 1, ρ+ 143B (TK−); lane 2, ρo 143B (TK−); lane 3, ρ+ 143B (TK−)γ; and lane 4, ρo LS180. The locations of the mtLeu tRNA representing the presence of mtDNA and DNA pol γ products from nuclear DNA are indicated. The two species for DNA pol γ are due to a difference in size of the two alleles, while the two products observed for mtLeu tRNA are believed to be the heavy- and light-strand sequences.

**Figure 2.** Ribonuclease protection assay analysis of DNA pol γ message levels in 143B (TK−) ρ+ (lanes 1, 2 and 7) and 143B (TK−) ρo (lanes 3–6, 8 and 9) cells. Ribonuclease protection on 20 μg of total cell RNA was performed as indicated in Materials and Methods. The first and second lanes of each pair for the DNA pol γ (lanes 1–6) correspond to protection with 2 and 4 ng of probe respectively. Lanes 7–9 show protection with 0.2 ng of the β-actin probe as a control. Lanes 10 and 11 are yeast RNA controls with the DNA pol γ or β-actin probes respectively, and lanes 12 and 13 correspond to ~0.25 ng of the undegraded DNA pol γ probe and 0.025 ng of the undegraded β-actin probe respectively.

**Analysis of DNA polymerase γ protein in 143B (TK−) cells**

DNA polymerase γ protein levels were analyzed in 143B (TK−) ρ+ or ρo cells. Consistent with our expression studies, western blotting analysis revealed that overall levels of DNA polymerase γ protein appeared equivalent in whole-cell extracts from both ρ+ and ρo 143B (TK−) cell lines (Fig. 3). A ~140 kDa species was present at equal levels in ρ+ and ρo cells (arrowhead, Fig. 3, lanes 1 and 2 respectively). Western blot analysis with fractionated protein species were more abundant in extracts from ρo cells, such as a protein ~100 kDa as well as a few lower molecular weight protein species (data not shown). However, levels of all of these proteins were immunoreactive with the pre-immune serum (Fig. 3, lanes 3 and 4). Previous studies have shown that the levels of certain proteins are elevated in ρo cells as compared with their parental counterparts (13). Equivalent amounts of protein were present in ρ+ and ρo cell extracts as revealed by nearly identical levels of actin protein (Fig. 3, lower panel).

Since ρo cells represent a long-term depletion of mtDNA, we were curious whether or not short-term depletion of mtDNA would exhibit any noticeable effect on levels of nuclear-encoded proteins involved in mtDNA copy number control, such as mtDNA polymerase γ and mitochondrial transcription factor A (mtTFA). Although EB is toxic to cells after approximately three cell generations (26), exposure to relatively low doses of EB for
short periods of time (a few days) results in reversible depletion of mtDNA (9).

We decided to emulate this paradigm in order to examine the presence of mtDNA polymerase γ in such cells. Human HeLa cells were treated with low concentrations of EB (20–100 ng/ml) for 3 days and then subjected to immunocytochemical analysis (Fig. 5). A similar cellular distribution pattern of mtDNA polymerase γ was apparent in untreated HeLa cells (Fig. 5a) as compared with 143B (TK−) cells (Fig. 4). Moreover, EB-mediated depletion of mtDNA in these cells had no significant effect on levels of the mtDNA polymerase γ with 20 ng/ml EB (Fig. 5b) or 100 ng/ml EB (Fig. 5c). This was in notable contrast with mtTFA, a protein which is required for accurate transcription of mtDNA and thus is likely a major regulator of mtDNA copy number (27). The presence of mtTFA closely mirrors the mtDNA status of the cell (28). We observed that either 20 or 100 ng/ml EB markedly depleted HeLa cells of mtTFA (Fig. 5e and f respectively). Depletion of mtDNA in these same cells was confirmed with triple-label confocal microscopic analysis, in which the incorporation of BrdU in mtDNA could be simultaneously measured (Davis and Clayton, submitted).

**DISCUSSION**

Mitochondria are semi-autonomous organelles containing a resident genome which encodes ribosomal RNAs, transfer RNAs and proteins, all of which function within the organelle. However, all mammalian mitochondrially-encoded proteins function as components of the respiratory chain assembly. The processes of replication and transcription of the mitochondrial genome, therefore, are accomplished entirely through the activities of nuclear-encoded enzymes. In this study, we have begun to analyze issues relating to communication between the nucleus and the mitochondrion, two distinct but interdependent organelles. ρ− cells serve as a model system in which to investigate potential changes in nuclear-encoded genes involved in mtDNA copy number control as a function of the presence or absence of mtDNA. Our results demonstrate that cells which have lost mtDNA either recently or chronically continue to express the DNA pol γ gene which encodes the mtDNA polymerase γ protein responsible for synthesizing mtDNA.

Previous studies in both yeast and mammalian cells have addressed the issue of potential nuclear compensatory responses in ρ− cells which are completely lacking in mtDNA (13–16,18).
These works have demonstrated variable changes in the expression of genes encoding documented regulators of mitochondrial function (13,16,28) or genes encoding proteins involved in cellular roles heretofore unrelated to mitochondrial function (13–16,18). It remains unclear what the functional implications of these changes are within the mitochondrion, since, at least in mammals, ρ0 mitochondria appear grossly normal and maintain a membrane potential.

We have shown that mtDNA polymerase γ is expressed and translated in the absence of mtDNA, and hence, in the absence of mtDNA replication. A paucity of nuclear genes encoding regulators of mtDNA copy number have been cloned in mammalian cells (6; Ropp and Copeland, submitted; Garman and Clayton, unpublished observations). Of these, mtTFA is a mitochondrially-imported transcription factor which is absolutely required (at least in vitro) for specific transcription initiation at mammalian mitochondrial promoters (27). Since in mitochondria, replication and transcription are functionally linked (2), this protein is likely a major regulator of mtDNA copy number. Previous studies have demonstrated that the stability and/or activity of mtTFA is tightly associated to the presence of mtDNA within the cell, since cells depleted of mtDNA are characterized by the absence of detectable levels of mtTFA protein (28). Interestingly, however, the mtTFA gene is expressed at normal levels in ρ0 cells (28). Our data corroborate those results, since we observed that even with low dose EB-mediated short-term depletion of mtDNA, mtTFA protein levels dropped severely. It thus appears that the activity of mtTFA is regulated stringently at a translational level.

In contrast, there appears to be no such control exerted over the mtDNA polymerase γ protein. Although the DNA pol γ gene is also expressed at normal levels in ρ0 cells, levels of mtDNA polymerase γ protein are indistinguishable from those in ρ+ cells. Furthermore, we observed that the subcellular distribution of this enzyme in ρ0 cells closely resembles that of the parental ρ+ cell line. These results are consistent with at least two possibilities. First, differences in protein levels between the two proteins may simply be an issue of stability. MtTFA, a member of the HMG box class of proteins, avidly binds DNA and may require such an interaction to maintain its stability within the cell. A second possibility is that mtDNA polymerase γ may play some as yet undefined additional role. By virtue of double-label confocal microscopy, we have detected the mtDNA polymerase γ protein only within mitochondria of the intact human cells used in this study. Previous studies employing biochemical fractionation techniques have identified nuclear (29–31) or extramitochondrial (32) forms of DNA polymerase γ. However, it is presently unclear whether or not such proteins are genetically distinct from the mtDNA polymerase γ, since those reports preceded the recent cloning of this gene. At the moment, the
concept that DNA polymerase γ plays any other role in the cell other than mtDNA synthesis remains, at best, speculative. However, we cannot rule out the possibility that the DNA polymerase γ protein might be maintained in \( \rho^0 \) cells to assist in maintaining the structural integrity of the \( \rho^0 \) mitochondrial membrane, although this appears unlikely since mtDNA polymerase γ is not among the most abundant proteins within the organelle.

Nonetheless, it is clear from these results that there appears to be no effective feedback mechanism to control the presence and presumed activity of mtDNA polymerase γ protein in the absence of mtDNA. Since \( \rho^0 \) cells have been depleted of mtDNA for hundreds of generations, it would appear extremely uneconomical for the cell to continue to produce and maintain a protein which it does not use. It is interesting to note, however, that mtDNA polymerase γ has been detected in sea urchin sperm, in which mtDNA replication does not occur (33). We do not know whether a similar scenario is operative in \( \rho^0 \) cells of \( S.cerevisiae \), since although the gene which encodes mtDNA polymerase γ in the yeast \( S.cerevisiae \) (MIP1) has been cloned (34), analogous experiments to analyze for the presence of this protein in the absence of mtDNA have not yet been reported. The reciprocal experiment, expectedly, gave unequivocal results: chromosomal disruption of the MIP1 gene resulted in complete loss of mtDNA in \( S.cerevisiae \) cells (34).

Our results do not implicate mtDNA polymerase γ as a major regulator of mtDNA copy number, and in fact strengthen the notion that other nuclear-encoded proteins such as mtTFA might play a significant role in that capacity. Potential differences in \( \rho^0 \) cells of \( S.cerevisiae \) in the abundance, activity or stability of other auxiliary proteins presumably involved in mtDNA replication such as mitochondrial single-stranded DNA-binding proteins (mtSSBs), primases and helicases, have not been measured. With the exception of human SSB (35), none of these nuclear genes has been cloned in human cells. The very recent cloning of DNA polymerase γ from the yeast \( Schizosaccharomyces pombe \) (36) and various types of vertebrate cells (Ropp and Copeland, submitted) should herald the beginning of the identification and characterization of some of these other proteins which function in the mechanics of mammalian mtDNA replication.

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